Article

Chinese Science Bulletin

Hydrobiology

June 2013 Vol.58 No.16: 1906–1911
doi: 10.1007/s11434-012-5643-8

Effects of dietary *tert*-butylhydroquinone on domoic acid metabolism and transcription of detoxification-related liver genes in red sea bream *Pagrus major*

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Received May 12, 2012; accepted October 26, 2012; published online February 28, 2013

Domoic acid (DA) is a neuroexcitatory amino acid that is produced by Pseudo-nitzschia during harmful algal blooms (HAB). Accumulation of DA can be transferred through food chain and cause neuronal damage in marine animal and in human. Like other algal toxins, DA was suggested to increase the oxidative stress and increase the detoxification-related gene expression in fish. The widely used food antioxidant, *tert*-butylhydroquinone (tBHQ), was known to induce a wide range of antioxidative potentials such as elevation of the glutathione levels and glutathione S-transferases (GSTs), via the activation of antioxidant response elements (AREs). In this study, the influences of dietary tBHQ on domoic acid (DA) metabolism and detoxification-related gene transcription were investigated both *in vivo* and *in vitro*. Oral administration of tBHQ resulted in significant decreases of DA accumulation of liver tissues in which red sea bream were fed with a single dose of 10 mg DA and 100 mg tBHQ per kg body weight per fish. Real-time PCR further revealed that the mRNA levels of *AHR*/*ARNT*/*CYP1A1*/*GSTA1*/*GSTR* were up-regulated in the above liver tissues at 72 h post tBHQ treatment. In consistence, tBHQ exposure also resulted in increased mRNA transcription of *GSTA1*, *GSTA2* and *GSTR* in cultured red sea bream hepatocytes. Collectively, our findings in this research suggested that the dietary intake of tBHQ accelerated DA metabolism in fish, through mechanisms involving altered transcription of detoxification-related liver genes.

tert*-butylhydroquinone, domoic acid, toxin metabolism, detoxification gene transcription, *Pagrus major

Citation: He S, Liang X F, Shen D, et al. Effects of dietary *tert*-butylhydroquinone on domoic acid metabolism and transcription of detoxification-related liver genes in red sea bream *Pagrus major*. Chin Sci Bull, 2013, 58: 1906–1911, doi: 10.1007/s11434-012-5643-8

Domoic acid is an excitatory neurotoxin produced by marine diatoms of the genus Pseudo-nitzschia. It is responsible for a neurotoxic disease called amnesic shellfish poisoning (ASP) in human and marine mammals following consumption of DA (domoic acid)-contaminated fish. Symptoms of ASP include short-term memory loss, brain damage and death in severe cases. During harmful algal blooms, accumulation of high concentration of DA has been reported for a number of marine species such as types of cockles (*Cerastoderma edule*), crabs (*Cancer magister*), furrow shell (*Scrobicularia plana*), mussels (*Mytilus edulis*), razor clams (*Siliqua patula*) and scallops (*Pecten maximus*) [1–3]. Other fish species, such as anchovies and mackerel, have also been shown to accumulate DA, although at much lower levels compared with those found in shellfish [3]. A majority of DA uptake was excreted via kidney and bile in fish, with the remaining absorbed DA mainly detected in liver, heart, spleen and muscle [4]. Limited data was available on *in vivo* metabolism in fish, although previous studies suggested that the biotransformation and detoxification pathways of Phase I and/or Phase II xenobiotic metabolism enzymes (XMEs) were involved in DA metabolism [5]. Further investigations using intraperitoneal (I.P.) injection of DA in fish revealed that a receptor-mediated induction of

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phase I XMEs cytochrome P-450 1A1 (CYP1A1) gene expression was involved in the biotransformation and detoxification of DA [6,7].

tert-Butylhydroquinone (tBHQ) is an phenolic antioxidant that is widely used as a food-additive. Dietary intake of tBHQ was shown to alter the activities of the endogenous antioxidant enzymes through activation of Nrf2 in both mammals and fish. Induction of a wide range of antioxidative potentials such as elevation of the glutathione levels and GSTs, via the activation of ARE, was believed to be responsible for the protective effect of tBHQ [8]. Furthermore, Gharavi and El-Kadi [9], and Schreiber et al. [10] demonstrated that tBHQ could also induce Phase I XMEs, cytochrome P450 1A1 (*CYP1A1*) gene expression directly in an aryl hydrocarbon receptor (AHR)-dependent manner in murine and human hepatoma cells. However, only limited information is available about the potential influence of tBHQ on DA metabolism in fish. Red sea bream *Pagrus major* is a commercial carnivorous fish breeding in the sea water of the east coast of China, which is inevitably polluted with DA during harmful algal blooms. In this study, we evaluated the effect of tBHQ on DA metabolism and detoxification-related gene transcription both *in vivo* and *in vitro*. The results could contribute to a better understanding of the molecular basis for DA detoxification, and bring new insights into the DA metabolism mechanism in fish.

1 Materials and methods

1.1 Toxin and reagents

Domoic acid (DA) and tert-butylhydroquinone (tBHQ) were purchased from BBI (Markham, Ontario, Canada) and Sigma (St. Louis, MO, USA), respectively. All the primers used in this research were synthesized by Sangon (Shanghai, China).

1.2 Hepatocyte isolation and tBHQ treatment

Red sea bream *Pagrus major* (body weight about 500 g per fish) were obtained from Zhangpu fish farm (Fujian Province, China). Fish were then killed by a sharp blow on the head and livers were pooled individually. After extensive wash to eliminate as much blood as possible, hepatocytes were isolated by 0.25% trypsin digestion at 28°C for 40 min. Cells were suspended in Hanks buffer and washed with the same medium for three times. Isolated hepatocytes were maintained in 6-well petri dishes (~1.12×10^7 cells mL^{-1}) in DMEM containing 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 5% fetal bovine serum and cultured at 28°C, supplied with 5% CO_2. The cells were then exposed to 40 μmol L^{-1} or 60 μmol L^{-1} tBHQ or media only (control treatment) for 24 h [11]. Every treatment was repeated four times. The number of cells was counted and cell viability was determined for each sample using Trypan blue exclusion at each time point before quantification of gene expression.

1.3 Rearing of red sea bream *Pagrus major* and exposure scenario

Red sea bream *Pagrus major* (16.92±4.28 g) were obtained from Zhangpu fish farm (Fujian Province, China). Fish were maintained in aquariums with continuous system of sea water filtration and aeration at constant temperature (26±2°C). Fish were fed with commercial fish diet at a rate of 2% of body weight twice (9∶00 and 15∶00) a day.

The red sea bream (n=20) were fasted for 24 h before oral gavaging of 10 mg kg^{-1} body weight (bwt) of DA and 100 mg kg^{-1} bwt tBHQ (within 200 μL total volume, mixed in semi-liquid feed of ground frozen fish and shrimp) using 16# bulb-tipped gavage needles [11]. The control fish were fed with tBHQ-free diet containing DA only. The fish were maintained in the above mentioned aquaria system. At each time point, five fish in each group were sacrificed at 24 and 72 h post treatment and the liver tissues were collected, respectively. The amount of DA in the liver tissues was determined.

1.4 Total RNA isolation from red sea bream hepatocytes and liver tissues

Total RNA was isolated from cultured hepatocytes or liver tissues using SV Total RNA Isolation System (Promega, USA), as described in the manufacturer's instruction. The extracted RNA was resuspended in 50 μL RNase-free water and quantified using Eppendorf Biophotometer (Germany). Samples were stored at −80°C before use.

1.5 Quantification of the mRNA of *AHR*, *ARNT*, *CYP1A1*, *GSTA1*, *GSTA2*, *GSTR and HSP70* by Real-Time PCR

The relative mRNA levels of *AHR*, *ARNT*, *CYP1A1*, *GSTA1*, *GSTA2*, *GSTR*, *Hsp70* and beta-actin in the hepatocytes and liver tissues of red sea bream were determined by RT-PCR. Gene-specific primers were designed with the Primer Designer Software 2.0, based on the cDNA sequences in the GenBank (Accession Nos. AB197788, FJ231886, EU107275, AB158410, AB158411, AB158412, AY190703, and AY190686, respectively). The sequences of the gene-specific primers and the sizes of PCR products are shown in Table 1. Beta-actin was amplified in parallel as an internal control. Reverse transcription was performed with oligo$(dT)_{20}$ primer using First Strand cDNA Synthesis Kit (Toyobo, Japan). The SYBR® Premix Ex Taq™ Kit (TaKaRa, Japan) was used for Real-Time PCR on a Chromo4 Real-Time Detection System (MJ Research, Hercules, CA, USA) according to the manufacture's instructions. Each PCR reaction consisted of 10 μL 2× SYBR Mix, 0.4 μmol L^{-1} forward primer, 0.4 μmol L^{-1} reverse primer, cDNA equivalent

Table 1 The sequences of primers for Real-Time PCR in red sea bream *Pagrus major*

Name of primer	Sequence of primer (GenBank accession Nos.)	Size of PCR product (bp)
FQ-PM-AHR01F	5′-CTATTCTCCATTGCTATGC-3′ (AB197788)	200
FQ-PM-AHR02R	5′-ATCATATCAGCTGCATGG-3′ (AB197788)	
FQ-PM-ARNT01F	5′-GGAGTATCATTAACAGACG-3′ (FJ231886)	187
FQ-PM-ARNT02R	5′-TGCAGCGGTGGTCTATGA-3′ (FJ231886)	
FQ-PM-CYP1A01F	5′-ATGAGAACTCCAATGTCCA-3′ (EU107275)	197
FQ-PM-CYP1A02R	5′-AAGGGTAAGTTGGGTTT-3′ (EU107275)	
FQ-PM-GSTA101F	5′-GCTACCTTCCAGTGTTCG-3′ (AB158410)	199
FQ-PM-GSTA102R	5′-TTCAGGAACTCGTCGATG-3′ (AB158410)	
FQ-PM-GSTA201F	5′-TGGAATCCTTGGCGACTT-3′ (AB158411)	196
FQ-PM-GSTA202R	5′-GTGAGGAGTGTACCAGAAC-3′ (AB158411)	
FQ-PM-GSTR01F	5′-CTGGCTGATGTGTGTGTT-3′ (AB158412)	203
FQ-PM-GSTR02R	5′-TGTTCCAGTGTGTCAGCATC-3′ (AB158412)	
FQ-PM-HSP7001F	5′-ACCTCTTCCGTGGCACCTT-3′ (AY190703)	195
FQ-PM-HSP7002R	5′-CTCCGTAGGCCACAGCTTCAT-3′ (AY190703)	
FQ-PM-ACT01F	5′-AATCGCCGCACTGGTTGTTG-3′ (AY190686)	217
FQ-PM-ACT02R	5′-CACAATACCGTGCTCGAT-3′ (AY190686)	

to that generated from 1 μg total RNA. ddH_2O was used to adjust the total volume of each reaction to 20 μL. Melting curve analysis of PCR products was performed at the end of each PCR reaction to confirm the specificity. For every sample, quantitative PCR was performed three times. PCR settings for all reactions were 95°C for 3 min, followed by 45 cycles at 95°C for 20 s, 57°C for 20 s, 72°C for 35 s. Having confirmed the amplification efficiency of each target gene, the relative quantifications of target gene expression were calculated with the delta Ct method using the Opticon Monitor software 2.03 Version (MJ research, USA). The relative mRNA levels of liver GSTs were expressed as the ratio of GSTs/beta-actin (%).

1.6 DA extraction and quantification by ELISA

Fresh red sea bream liver samples (1 g) were ground by sonication. Homogenates were extracted twice with 100% methanol for 30 min with sufficient mixing using a magnetic stirrer, followed by centrifugation at 4000 r min^{-1} for 10 min. The supernatant was concentrated on Sep-pak C18 cartridge (Waters), which was pre-washed with 100% methanol and water. The cartridge was then rinsed with 25 mL 20% methanol and was eluted with 5 mL 100% methanol. The elusions were air-dried and re-dissolved in 0.5 mL of 100% methanol∶water (1∶1, v/v). DA concentrations in these samples were quantified by immunoassay using EIA Domoic 2 h Kit (Groupe, Belgium). Triplicates of DA standards of six different concentrations and triplicates of each sample were included in each microtiter plate for measuring (TECAN Infinite 200, Austria). A standard curve was constructed and the average value of each triplicated samples was recorded for further analysis (Magellan software V 5.03).

1.7 Statistical analysis

Statistical analysis was performed with SPSS13.0 software. Significant differences were found by using one-way analysis of variance (ANOVA), followed by the *post hoc* test, after checking for data normality and homogeneity of variances. Differences were considered to be significant if $P<0.05$. Values were expressed as means±S.E. (n=5) for each group.

2 Results

2.1 The influences of tBHQ on the mRNA expression of *AHR*, *ARNT*, *CYP1A1*, *GSTA1*, *GSTA2*, *GSTR* and *HSP70* in cultured hepatocytes

Due to the antioxidative potentials of tBHQ, the transcription of the endogenous antioxidant enzymes including AHR, ARNT, CYP1A1, GSTA1, GSTA2, GSTR and HSP70 was analyzed in cultured hepatocytes. Slight decrease of cell numbers was observed in the cultured red sea bream hepatocytes when exposed to tBHQ. The cell viabilities were maintained ⩾96% throughout the experiment in both control cells and cells exposed to tBHQ. Compared with that in the control cells, tBHQ treatment, at both 40 and 60 μmol L^{-1}, significantly induced GSTs transcription in hepatocytes.

The mRNA levels of *GSTA1*, *GSTA2* and *GSTR* were up-regulated by 1.22, 1.33 and 1.09-fold on 40 μmol L^{-1} tBHQ and by 1.13, 1.28 and 1.58 fold on 60 μmol L^{-1} tBHQ, respectively. The expression of AHR was also slightly induced by tBHQ treatment. However, no further induction was observed with tBHQ treatment at higher concentration. In contrast, the transcription of CYP1A1 was slightly decreased in cultured hepatocytes that treated with 40 μmol L^{-1} tBHQ and was dramatic decreased (P<0.05) in cultured hepatocytes treated with 60 μmol L^{-1} tBHQ. The influences of tBHQ on the transcription of ARNT and Hsp70 were debatable, with controversial results obtained in cultured hepatocytes treated with tBHQ treatment at different concentrations (Figure 1).

2.2 The influences of tBHQ on AHR, ARNT, CYP1A1, GSTA1, GSTA2, GSTR and HSP70 transcription *in vivo*

Due to the critical roles GSTs play in DA detoxification and the significantly increased GSTs expression in cells exposed to tBHQ *in vitro*, the influences of tBHQ on mRNA expression of GSTs in the liver tissues of the red sea bream previously exposed to DA were determined using real-time PCR. Compared with others, the mRNA levels of *AHR* and *ARNT* were extremely low and were not presented in the same figure. The results showed that compared with the control group, oral gavaging tBHQ resulted in dramatic increase, about 2.87-fold, in the relative mRNA level of *GSTA2* in the liver tissue of the red sea bream 24 h after fed with tBHQ (Figure 2). However, no significant difference was observed on the *GSTA2* mRNA level between the control fish and the tBHQ fed fish at 72 h after DA treatment. In contrast, the

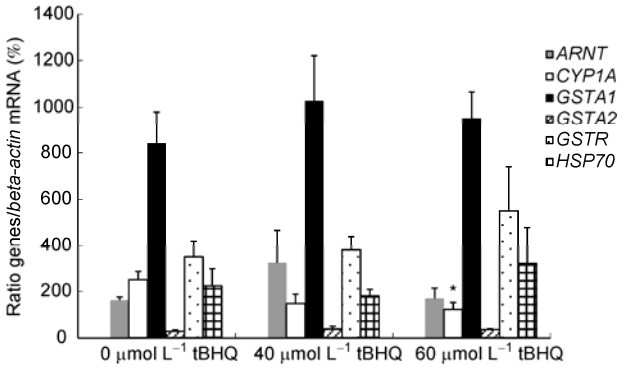

Figure 1 The influences of tBHQ on detoxification-related gene transcription *in vitro*. The relative mRNA abundance of *AHR*, *ARNT*, *CYP1A*, *GSTA1*, *GSTA2*, *GSTR* and *HSP70* in cultured red sea bream *Pagrus major* hepatocytes was determined by Real-Time PCR after exposure to 0, 40 and 60 μmol L^{-1} tBHQ for 24 h. The level of AHR was too low to be presented in the same figure, although slight induction of expression by tBHQ treatment was also observed. Columns represent the means of four repeats of each sample. Bars represent the standard errors. Significant changes (P<0.05) were found by using one-way analysis of variance (ANOVA), followed by the *post hoc* test, after checking for data normality and homogeneity of variances, and were indicated with stars (*).

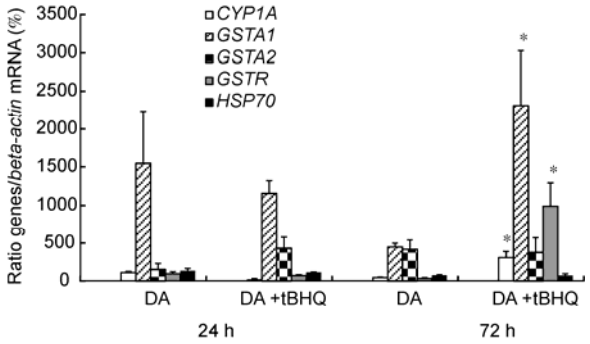

Figure 2 The influences of tBHQ on detoxification-related gene transcription *in vivo*. The relative mRNA abundance of *AHR*, *ARNT*, *CYP1A*, *GSTA1*, *GSTA2*, *GSTR* and *HSP70* in red sea bream *Pagrus major* liver tissues were determined by Real-Time PCR at 24 and 72 h after oral administration of 10 mg kg^{-1} DA and 100 mg kg^{-1} tBHQ. The mRNA levels of AHR and ARNT were very low and not presented in the same figure, while significant increases of their transcription were also observed at 72 h post tBHQ treatment. Columns represent the means of the data obtained in five fish for each treatment. Bars represent the standard errors. Significant changes (P<0.05) were found by using one-way analysis of variance (ANOVA), followed by the *post hoc* test, after checking for data normality and homogeneity of variances, and were indicated with stars (*).

mRNA levels of liver AHR, ARNT, CYP1A1, GSTA1 and GSTR were barely affected at 24 h after tBHQ treatment, although were significantly increased by 3.21, 1.94, 7.18, 5.17 and 30.21-fold, respectively, at 72 h after oral administration of tBHQ (P<0.05) (Figure 2). No detectable change was observed for *HSP70* mRNA transcription in fish liver at both time points. The fluctuation of these gene transcription might reflect the process of DA metabolism and excretion in the liver of red sea bream.

2.3 The influence of tBHQ on the accumulation of DA in liver tissues

A single dose of 2% of body weight commercial feed containing 10 mg kg^{-1} body weight (bwt) of DA and 100 mg kg^{-1} bwt tBHQ per fish was used to feed each red sea bream that had fasted for 24 h. The control fish were fed with tBHQ free diet containing DA only. Throughout the entire observation period of experiment, the liver tissues of red sea bream displayed unnoticeable pathological changes in regard to the shape and the color. Neither liver bleeding nor edema was observed with naked eyes in those fish by the time of sacrificing. The concentrations of DA in the liver tissues of red sea bream at 24 and 72 h after oral administration of tBHQ were shown in Figure 3. At 24 h post tBHQ treatment (accumulation period), the concentration of DA in liver tissues of the treatment group was 16.79±6.91 ng g^{-1} tissue, much lower than that of the control group (66.17±29.60 ng g^{-1} tissue) (P<0.05). However, at 72 h after oral administration of tBHQ (depuration period), the DA concentration in liver tissues of control group decreased dramatically (4.48±3.90 ng g^{-1} tissue) and no significant difference in DA accumulation in liver was observed between

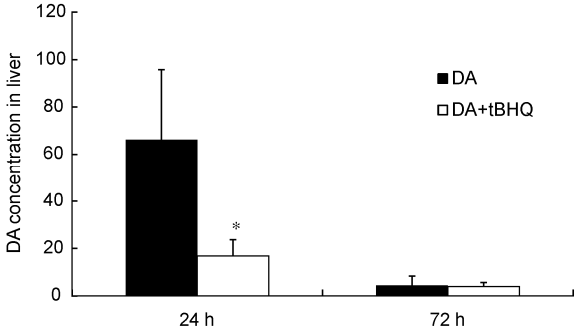

Figure 3 The effect of tBHQ on DA accumulation in liver. The DA concentrations in the liver tissues of red sea bream *Pagrus major* were determined at different time points after oral administration of 10 mg kg^{-1} DA and 100 mg kg^{-1} tBHQ. Columns represent the means of the data obtained in five fish for each treatment. Bars represent the standard errors. Significant changes ($P<0.05$) were found by using one-way analysis of variance (ANOVA), followed by the *post hoc* test, after checking for data normality and homogeneity of variances, and were indicated with stars (*).

the control group and the tBHQ treated group (3.90±1.71 ng g^{-1} tissue).

3 Discussion

Harmful algal blooms are globally increasing natural events and may cause sub-lethal effects such as increased stress behaviour, including lowered spontaneous activity, loss of appetite and reduced overall growth in human following consumption of contaminated seafood. Domoic acid is a water-soluble amino acid produced by Pseudo-nitzschia during HAB and causes excitoxicity/death in neuron cells by binding glutamate receptor irreversibly in both marine animals and human. It was shown in previous studies that short term toxin exposure reduced survival of larval fish [12]. In fish, orally administered DA could be absorbed from GI track and absorbed DA could be detected in plasma and various tissues within 15 min after DA exposure [13,14]. Absorbed DA was largely excreted via kidney and bile in the first 72–96 h, with remaining DA subjected to biotransformation to less toxic derivatives, a process yet to be clarified. For instance, like other algal toxins, DA increased oxidative stress and the activities of liver detoxification enzymes, including GPx, CAT and SOD in fish [15].

Previous studies showed that tBHQ could induce Phase II XMEs and exhibited anticarcinogenic effects in human and rat cells [16,17]. Exposure to tBHQ was shown to trigger the accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2) in nuclear, where they could form heterodimers with other transcription factors, such as Jun and small Maf [18]. These heterodimers could then bind the AREs of the 5′-regulatory region of several Phase II detoxification genes, including GSTs, and then activate the GSTs gene expression [19–22]. Our results in this study showed that tBHQ exposure significantly induced the transcription of detoxification-related genes in red sea bream hepatocytes using Real-Time PCR. The mRNA levels of *GSTA1*, *GSTA2* and *GSTR* were increased after tBHQ exposure in hepatocytes both *in vitro* and *in vivo*, indicating a role in inducing Phase II XMEs for tBHQ in red sea bream. It was in consistent with the proposed antioxidant protective role for tBHQ in fish [23]. In addition, Poulson et al. [24] had characterized a functional XRE and an ARE in rat GSTA2 and their proximity. Human GSTA1 and GSTA2 could be induced by AHR agonists in human hepatocyte cultures of some individuals [25,26]. Further studies are needed to determine whether the induced expression of Phase II XMEs (GSTA1, GSTA2 and GSTR) by tBHQ in red sea bream hepatocytes are due to multiple mechanisms linking AHR- and Nrf2-induced batteries [27].

The mRNA expression levels of *AHR* and *ARNT* were significantly increased in the red sea bream liver tissues at 72 h after oral administration of tBHQ. Not only Phase I genes (AHR and ARNT) but also Phase II genes (GSTA1, GSTA2 and GSTR) expression levels were induced significantly by tBHQ in red sea bream liver tissues, indicating that the metabolic adaptation of tBHQ might be coordinately regulated by a set of phases I and II XMEs. Further characterization of the response elements such as XREs and AREs in the regulatory region of these target genes may provide a genetic basis to understand AHR- and Nrf2-induced genes. The effect of tBHQ on Phase I XME expression, CYP1A, needs further investigation since controversial results were obtained in cultured hepatocytes and in liver tissues *in vivo*. We further investigated the potential influence of tBHQ on DA metabolism in red sea bream *in vivo*. A single dose of approximately 10 mg DA kg^{-1} fish was used for oral gavage, representing the environmentally relevant daily maximum dose of DA. No signs of neural toxicity and death were observed in our experimental fish. Comparing with the DA accumulation (66.17±29.60 ng g^{-1} tissue) in liver tissues of control group, tBHQ exposure significantly decreased DA accumulation (16.79±6.91 ng g^{-1}) within 24 h after tBHQ treatment. The results indicated that dietary intake of tBHQ could effectively stimulate the first phase of DA excretion in red sea bream liver, which could be the consequence of the elevated Phase II XMEs gene expression. However, no obvious difference in the DA concentration in liver tissues was observed between tBHQ treated fish and the control fish at 72 h after oral administration of tBHQ. It suggested that tBHQ may have very limited influence on the second phase of DA elimination.

In conclusion, our results in this study suggested that dietary intake of tBHQ can effectively stimulate DA metabolism, possibly by increasing the transcription of detoxification-related liver genes in fish. The metabolic adaptation may be consequences of coordinately regulated phases I and II XMEs.

We thank Dr. Jeffrey Silverstein for his helpful review of the manuscript. This work was supported by the National Natural Science Foundation of China (30670367), the National key Basic Research and Program of China (2007AA09Z437), and the Fundamental Research Funds for the Central Universities (2010PY010, 2011PY030).

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